Studies on ectomycorrhizae of pine. I. Production of volatile[1] organic compounds

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The mycorrhizal fungus *Boletus variegatus* Fr. was grown in pure culture and its production of volatile organic compounds studied. Maximal production of volatile organic compounds was associated with actively growing mycelia. The major volatile compounds identified in the culture filtrate were ethanol, isobutanol, isoamyl alcohol, acetoin, and isobutyric acid. Of these, isobutanol and isobutyric acid are known to possess fungistatic activity. Volatile organic compounds were also extracted from the whole root systems of *Pinus sylvestris* L. (Scots pine) seedlings grown in pure culture with and without the fungal symbiont. Infection of the roots by the fungus resulted in production and (or) accumulation of volatile compounds in concentrations two to eight times greater than that of non-inoculated controls. These compounds were identified by combined gas chromatography and mass spectrometry. They were primarily terpenes and sesquiterpenes. Volatile compounds produced by the mycorrhizal root system of Scots pine collected from a nursery were essentially the same as those obtained from the plants grown in pure culture. Many of these are known to be fungistatic compounds. A hypothesis has been proposed to explain a possible role of the host plant in disease resistance of mycorrhizal root systems to root pathogens and in the development of the symbiotic state.

Introduction

In many species of trees ectomycorrhizae are vital for the establishment of seedlings and for their continued development in soils low in nutrients. Zak (1964) suggested that ectomycorrhizal roots may be less susceptible to root pathogens than nonmycorrhizal roots. Recently Marx (1969*a*, *b*, 1970) and Marx and Davey (1969) demonstrated experimentally that ectomycorrhizae of pine can act as deterrents to *Phytophthora cinnamomi*. In this connection considerable attention has been given to the production of antibiotic substances by the fungal symbionts (Marx 1969*a*, *b*; Krywolap *et al.* 1964) and very little is known about the possible role of metabolites produced by the host in response to the infection by the fungal symbiont (Hillis and Ishikura 1969). Mycorrhizal fungi may stimulate host roots to produce or accumulate sufficient concentrations of substances inhibitory to root pathogens. This type of host reaction has been demonstrated in endomycorrhizae (Gäuman *et al.* 1960).

Almost all knowledge of the role of root metabolites in disease resistance pertains to nonvolatile substances (Schroth and Hildebrand 1964). Recently McDougall (1970) demonstrated that volatile materials made up 80% of all metabolites liberated into an acidic medium supporting roots of wheat plants. This is particularly relevant to Scots pine, since this plant generally grows in acidic soils (Wilde 1946). In addition pine roots are known to contain oleoresins rich in volatile constituents.

The objective of the present investigation was to identify and determine the relative amounts of various volatile organic compounds produced by (1) mycelium of *Boletus variegatus* Fr. in pure culture, (2) root systems of *Pinus sylvestris* L. in pure culture, (3) mycorrhizal root systems of *P. sylvestris* developed in aseptic cultures with *B. variegatus*, and (4) mycorrhizal root systems of *P. sylvestris* grown in a nursery.

Material and Methods

The strain of *Boletus variegatus* Fr. used in this investigation was isolated in 1969 and was kindly provided by Prof. Elias Melin of this institute. The fungus was maintained on Hagem malt extract agar (Norkrans 1950). In the growth experiments the fungus was grown in still cultures in 2-liter Fernbach flasks with 250 ml of medium II of Norkrans (1950) at 25°C in dark.

The inoculum was prepared as follows. Pieces of mycelium and Hagem agar about 2 mm square were transferred to 100-ml erlenmeyer flasks containing 20 ml of Norkrans medium II. The fungus was allowed to grow for 7 days. Mycelia from 30 of these flasks were pooled and homogenized with glass beads (Wikén *et al.* 1951) in 100 ml of sterile distilled water. Two milliliters of the mycelial suspension was used as inoculum for each Fernbach flask. Growth was measured as dry weight of

[1]The term volatile here denotes substances which may produce biological effects in the gas phase.

the mycelium in four replicate flasks. After the mycelia were harvested, the filtrate was used for determinations of pH and glucose and for extraction of volatile compounds. Glucose determinations were made with glucose oxidase according to Lindberg (1963).

In all the experiments redistilled ether was used as a solvent for extracting volatile compounds because of some of its desirable qualities: (1) ether has a boiling point of 34.6°C, facilitating extraction of compounds with a low boiling point, (2) ether extraction excludes the presence of sugars and water in the sample and thus reduces interference from these compounds in gas chromatography, and (3) ether is a solvent for molecules with lipophilic properties.

Aseptic Synthesis of Ectomycorrhizae

Seeds of *Pinus sylvestris* L. were washed thoroughly with distilled water and surface-sterilized by being shaken in 7% calcium hypochlorite for 5 min. The seeds were then rinsed several times with sterile distilled water and germinated on water agar at 20°C in dark. When the roots were 3–4 cm long, they were transferred to 500-ml wide-mouthed erlenmeyer flasks containing an autoclaved mixture of 150 ml screened terralite and 120 ml of the following medium: 0.5 g K_2HPO_4, 0.05 g $CaCl_2$, 0.025 g NaCl, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.25 g $(NH_4)H_2PO_4$, 1.2 ml Fe citrate (1 *M* Fe citrate in 1 *M* citric acid), 2.5 g glucose, and 5 µg thiamine brought to a final volume of 1 liter with distilled water. The pH was adjusted to 3.5 before autoclaving. The seedlings were then grown in the greenhouse under continuous artificial light (about 50 ft-c per plant) at 19°–22°C. After 2 months each flask was inoculated with a piece of mycelium about 4 mm^2 of *Boletus variegatus* grown on Hagem agar. The agar block was embedded in the terralite adjacent to the roots. At the same time an additional 30 ml of the sterilized medium described previously was added to each flask. Three months later inoculated and uninoculated (control) seedlings were extracted for volatile organic compounds. Before extraction random seedlings from the inoculated flasks were checked microscopically for well-developed mycorrhizal roots. In addition roots from inoculated and uninoculated controls were plated to check for contamination. In no case were any contaminating organisms detected.

Extraction of Volatile Organic Compounds from the Fungus Culture Filtrate

Redistilled diethyl ether (reagent quality) in a conventional type liquid/liquid extractor was used to extract 750 ml of culture filtrate for 17 h. At the end of the extraction, anhydrous Na_2SO_4 was added to the ether phase and allowed to stand overnight at 4°C. The ether in the extract was removed by fractional distillation through a short glass column packed with small glass cylinders. The residue was generally 3–6 ml.

Extraction of Volatile Organic Compounds from Roots

Thirty seedlings were removed from the flasks taking care not to injure the roots and making no effort to remove some of the terralite particles adhering to the roots. After excision at the root collar the whole root system of each plant was dropped directly into a soxhlet column containing a known quantity of redistilled ether. The combined root systems from the 30 plants were extracted for 11 h at room temperature with a total volume of 350 ml of ether. The volatile compounds were concentrated from this extract by the same method used for the volatile fungal metabolites. After extraction the roots were dried for weight determinations. The foregoing procedure was followed separately for both inoculated and uninoculated seedlings.

The main root of the inoculated plants had an average length of 50 cm per plant and the dry weight of the whole root system was about 2.5 times more (430 mg per plant) compared to the uninoculated controls (173 mg per plant). The main root of the controls had an average length of 40 cm per plant.

Nursery seedlings (1–2 years old) were obtained from Närlinge, Sweden. The seedlings were removed together with the surrounding soil and brought immediately to the laboratory and the root systems of eight seedlings were processed by the same method as the greenhouse material. The nursery material was also checked at random for mycorrhizal development.

Analyses

Compounds were fractionated and components tentatively identified by gas chromatography on a Perkin-Elmer 880 chromatograph equipped with a flame ionization detector. Samples were injected with Hamilton syringes (fungus extract 2 µl and root extracts 10 µl). Column: 3.5 m × 0.475 cm Ø copper tube packed with 20% Carbowax 20 M on chromosorb (W-AW). N_2 carrier gas flow rate was about 50 ml/min. For fungus extracts column temperature was 150°C isothermal and for root extracts 110°C isothermal. After the volatile compounds were separated, most of the individual peaks were trapped in chilled acetone (analytical reagent) by several successive runs of the test sample. The collected fractions as well as the total sample were further identified in a combined gas chromatograph and mass spectrometer (LKB 9000) at the Svenska Institutet för Konserveringsforskning, Göteborg, Sweden.

Results and Discussion

Production of Volatile Organic Compounds by Boletus variegatus in Liquid Cultures

Maximal production of volatile organic compounds occurred at 17 days of growth. This corresponded to the highest rate of mycelial growth (Δ growth/time) and pH change (Δ pH/time) in the medium (Fig. 1). The mycelial dry weight increased beyond 17 days, maximal dry weight (2.2 g per flask) being obtained on the 25th day. The decrease in the production of volatile compounds beyond 17 days was not due to lack of carbon, since 68% of the glucose was available in the medium at that time. The economic coefficient (mg dry matter produced per 100 mg glucose consumed) was 44.0. The

major volatile substances produced by *B. variegatus* were ethanol, isobutanol, isoamyl alcohol, acetoin, and isobutyric acid (Fig. 2, Table 1). Of these, vapors of 0.025 *M* isobutanol inhibited the growth of *Phytophthora cinnamomi* and *Fomes annosus* while vapors of 0.01 *M* isobutyric acid inhibited *P. cinnamomi*, *F. annosus*, *Rhizina undulata*, and *Trichoderma viride* (Krupa and Nylund).[2]

Earlier, isobutanol had been demonstrated to inhibit the vegetative growth of *Pestalotia rhododendri* (Norrman 1968). Similarly isobutyric

[2]Manuscript in preparation.

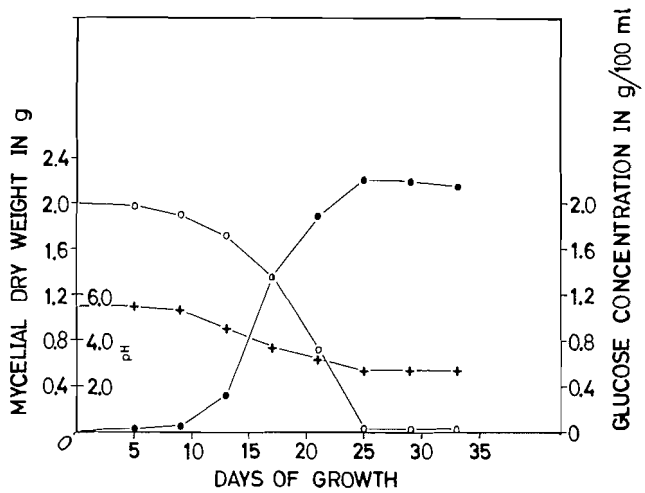


FIG. 1. Growth of *Boletus variegatus* in liquid cultures in Norkrans medium II (250 ml/flask) at 25°C in dark. Closed circles = mycelial dry weight, open circles = glucose content in the medium, + = pH.

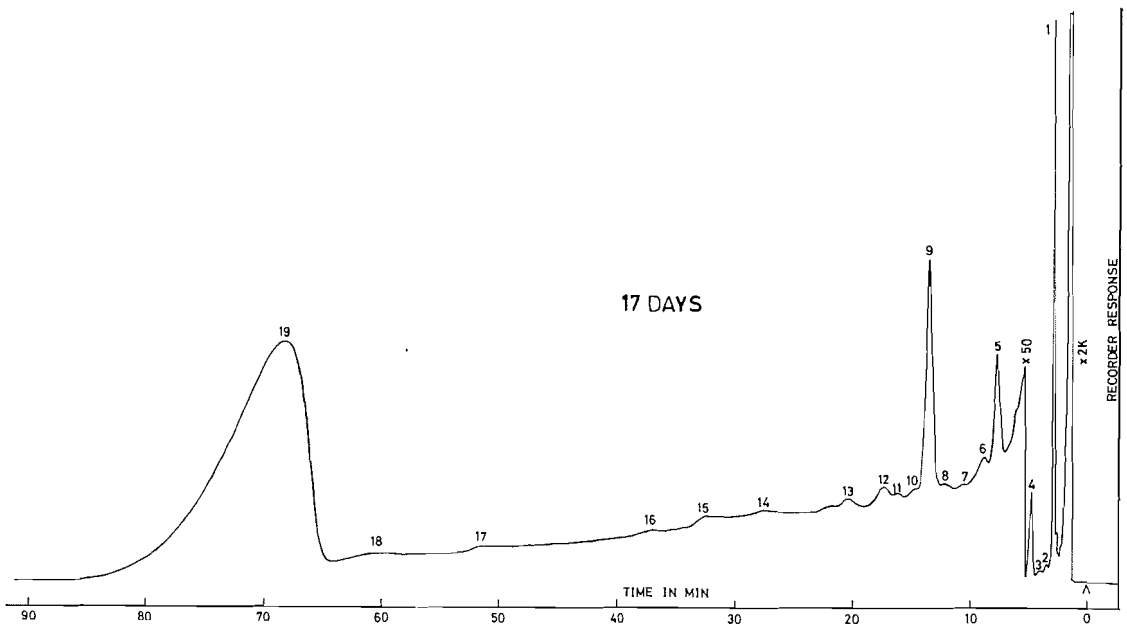


FIG. 2. Gas chromatogram of volatile organic compounds produced by *Boletus variegatus*. Column: 20% Carbowax 20 M on Chromosorb (W-AW); column temperature: 150°C isothermal.

TABLE 1

Major volatile compounds produced by *Boletus variegatus* in Norkrans medium II after 17 days of growth

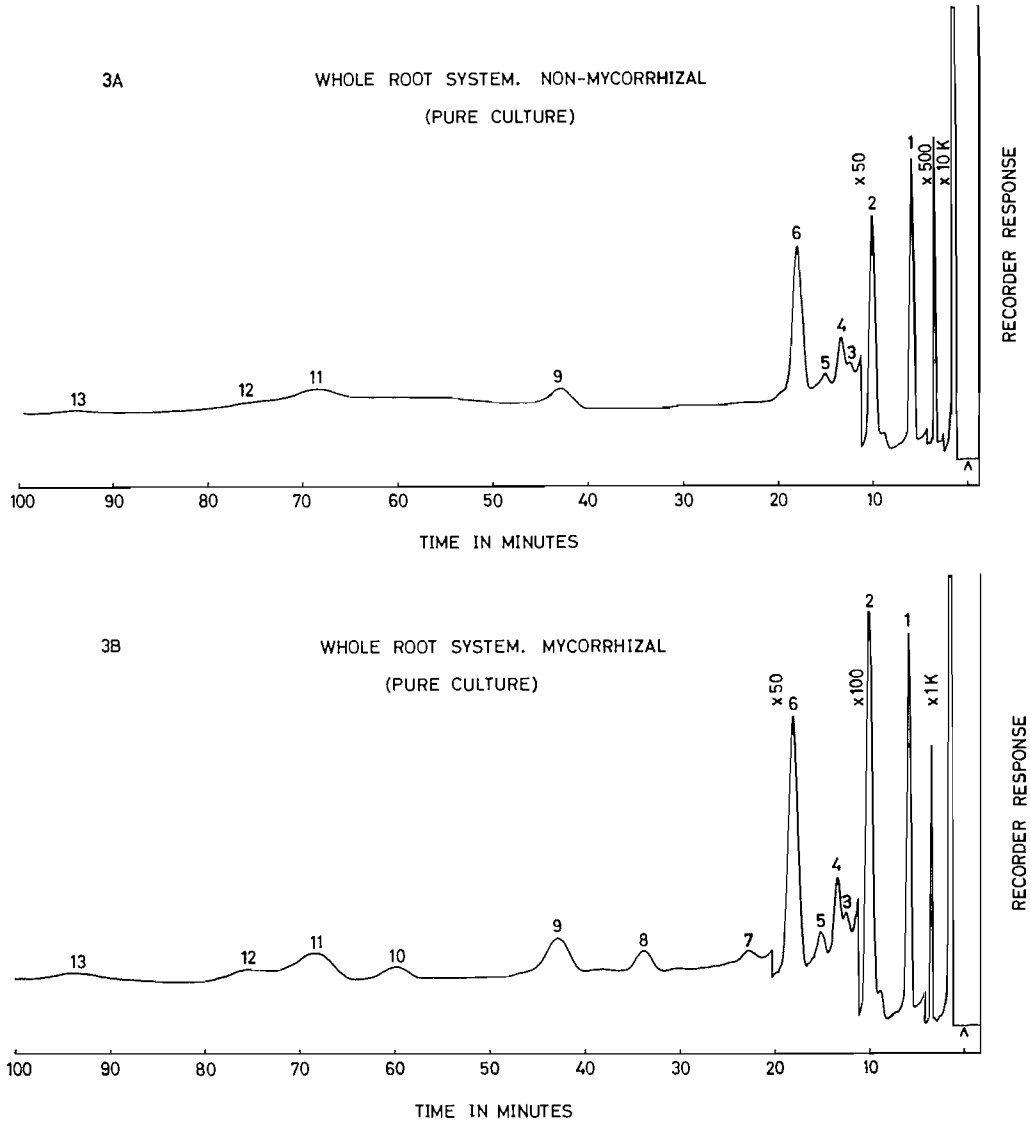
Fig. 2 peak No.	Identity of the compound
1	Ethanol
4	Isobutanol
5	Isoamyl alcohol*
9	Acetoin
19	Isobutyric acid

*Isoamyl alcohol was identified by its retention times on 20% Carbowax 20 M and FFAP columns. All the other peaks were identified by combined GC–mass spectrometry.

acid or its related fatty acids inhibited growth of *Fomes annosus* and *Phymatotrichum omnivorum* (Baechler 1939; Riegler and Greathouse 1940).

Production of Volatile Organic Compounds by Ectomycorrhizal Root Systems of Scots Pine

Nonmycorrhizal and mycorrhizal root systems contained essentially the same major volatile compounds (Figs. 3A, 3B). Generally infection of the roots by the mycorrhizal fungus resulted in two- to eight-fold increase in concentrations of all the individual substances over uninoculated controls (Table 2). These were primarily



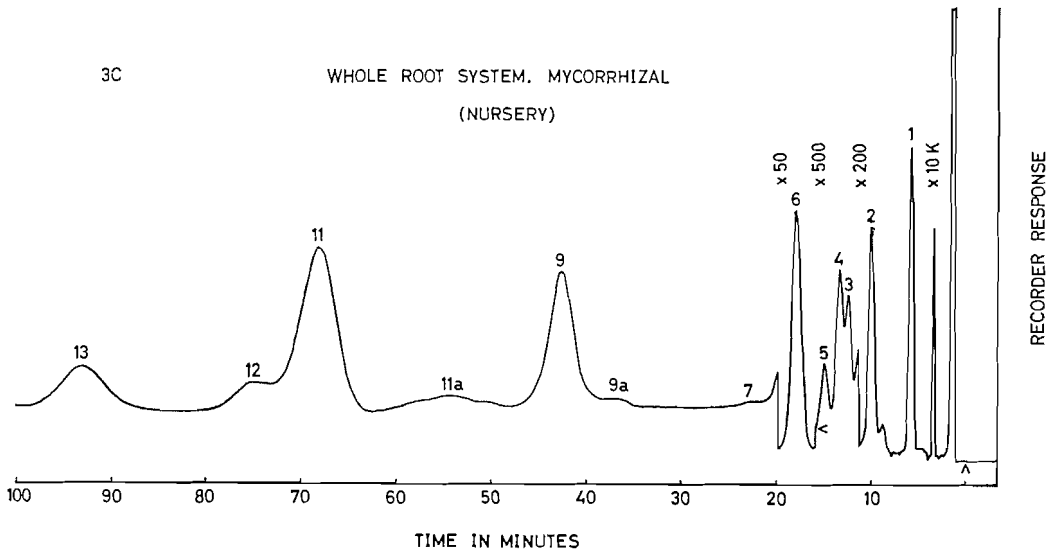


FIG. 3. Gas chromatographic analyses of volatile organic compounds produced by (A) nonmycorrhizal root system of *Pinus sylvestris* grown in pure culture, (B) mycorrhizal root system of *P. sylvestris* grown in pure culture with *Boletus variegatus*, and (C) mycorrhizal root system of *P. sylvestris* collected from a nursery. Column: 20% Carbowax 20 M on Chromosorb (W-AW); column temperature: 110°C isothermal. For the identities of individual peaks refer to Table 2.

monoterpenes and sesquiterpenes. Of the monoterpenes α-pinene, 3-carene, and terpinolene were the chief products. 3-Carene increased most (four-fold) in the inoculated over the uninoculated controls. Of the smaller constituents γ-terpinene increased eight-fold in the presence of the fungal symbiont. There were also several terpenoids and sesquiterpenes in higher amounts in the mycorrhizal root system. These substances are yet to be identified because of the complexity of their structure. Comparison of Figs. 3B and 3C indicates that the production of volatile compounds was remarkably similar qualitatively in the greenhouse and the nursery materials. Peak No. 8 (Fig. 3B) was an exception, being found exclusively in the synthetic mycorrhizal root system. The increased concentrations of terpenes and sesquiterpenes in the mycorrhizal material was associated with a similar increase in the amounts of phenolic substances (details to be published separately).

The foregoing results differed from those reported earlier by Hillis and Ishikura (1969) for *Pinus radiata* in their study of non-volatile compounds in mycorrhizal and nonmycorrhizal roots. These authors obtained more non-volatile extractives in the nonmycorrhizal than in the mycorrhizal portions of the root systems. This could have been due to differences in age between the types of tissues used in their study, the mycorrhizal tissue being composed of younger portions of the root system. Furthermore, these authors studied material collected from nurseries where organisms other than the fungal symbionts could also be members of the rhizosphere and the rhizoplane resulting in the observed quantitative differences caused by multiple host response. To avoid these sources of error, in the present investigation the plants were grown under totally sterile conditions and the whole root system with and without the fungal symbiont was sampled. Subsequently roots with mycorrhizae from a nursery were analyzed to determine the relevancy of the substances identified in the synthetic material.

All the substances identified in the synthetic mycorrhizal root system were strictly of host origin. The metabolites produced by the fungal symbiont in pure culture were not detected. In subsequent preliminary analyses of the synthetic mycorrhizal root systems as well as the media supporting them, metabolites produced by *B. variegatus* were detected in small quantities. The inability to detect the fungal metabolites in the analyses of the synthetic mycorrhizal material could have been due to the insufficient amounts

of actively growing mycelia of the fungal symbiont on the root surface to permit their detection. Results from the pure culture studies indicate that maximal production of volatile compounds is associated with actively growing mycelia.

Many of the monoterpenes identified in the ectomycorrhizal root system are constituents of the tree oleoresin. Oleoresins play an important role in the resistance of wood to decay fungi (Rishbeth 1951). Volatile oleoresin components from *Pinus ponderosa* inhibit the vegetative growth of *Fomes annosus* and four *Ceratocystis* species (Cobb *et al.* 1968). Some sesquiterpenes contribute to the general resistance mechanism in plants (Goodman *et al.* 1967, p. 198).

The forementioned increased production and (or) accumulation of terpenes and phenolic substances in the ectomycorrhizal root system would seem to be due to a non-specific response of the host. Wounding (Cobb *et al.* 1968) as well as root pathogens (Rishbeth 1951) induce a similar host response.

In conclusion the following hypothesis is proposed to explain one of the roles of the host in the disease resistance of ectomycorrhizal root systems (Fig. 4). The host roots produce stimulatory substances, volatile (oxidation products of fatty acids etc.) and non-volatile (vitamins, M factor etc., Melin 1963) for the growth of mycorrhizal as well as nonmycorrhizal fungi. The mycorrhizal fungi on the other hand produce substances antibiotic against root pathogens resulting in the growth inhibition of those fungi. This subsequently leads to the entry of the mycorrhizal fungi into the host root. The response of the host to this infection is non-specific, being similar to a wound response. Presumably the mycorrhizal fungi determine the rate of the host response. Host response results in the increased production and (or) accumulation of native volatile (terpenes etc.) and non-volatile (phenols etc.) substances. These substances, when present in sufficient concentrations may restrict the growth of the mycorrhizal fungi within the host tissue, resulting finally in the symbiotic state. At the same time the root pathogens are also inhibited. The monoterpenes and some of the sesquiterpenes would seem to have a far-reaching effect into the rhizosphere due to their volatility, thus slowing down the growth of the root pathogens. Phenolic substances and some of the sesquiterpenes would be important within the host tissue. In addition to these and the non-volatile antibiotic substances reported earlier by others for mycorrhizal fungi, volatile products of the actively growing fungus

TABLE 2

Volatile organic compounds in ether extracts of mycorrhizal and nonmycorrhizal root systems of *Pinus sylvestris* grown in pure culture with and without *Boletus variegatus*

Fig. 3B peak No.	Major constituent	Relative amounts A Nonmycorrhizal	B Mycorrhizal	B/A
1	α-Pinene	270.00	780.00	2.9
2	3-Carene	290.00	1200.00	4.1
3	Limonene	0.50	2.00	4.0
4	β-Phellandrene	5.00	18.00	3.6
5	γ-Terpinene	1.00	8.00	8.0
6	Terpinolene	30.00	122.0	4.0
7	Allo-ocimene?		2.00	2.0
8	Unknown		6.00	6.0
9	α-Longipinene	8.00	17.00	2.1
10	Unknown		4.00	4.0
11	Longifolene	6.00	16.00	2.7
12	Sesquiterpene	Trace	2.00	2.0
13	Sesquiterpene	Trace	3.00	3.0

NOTE: Relative amounts were calculated as follows. Concentration of γ-terpinene in the nonmycorrhizal material = 1. Concentrations were calculated from the dry weight of the area of the individual peaks in a xerox copy of the original chromatogram. These values were corrected for differences in the dry weight of the root tissues and the final volume of the extract.

Minor constituents identified: camphene, β-pinene, sabinene, myrcene, α-terpinene, *p*-cymene, tertiary butyl anisole?, 4-terpinenol?, $C_{10}H_{14}O$ terpinoid, $C_{10}H_{16}O$ terpinoid, $C_{10}H_{18}O$ terpinoid, and $C_{15}H_{24}$ sesquiterpene. All the minor constituents were found in mycorrhizal but were barely seen in the nonmycorrhizal root systems.

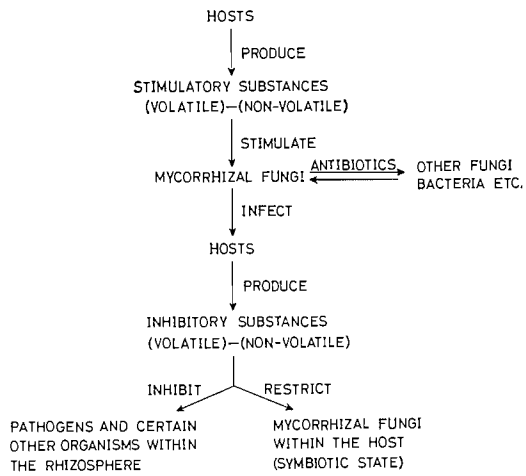


FIG. 4. A proposed mechanism for the role of the host plant in the disease resistance of ectomycorrhizae.

mycelia such as isobutanol and isobutyric acid would at least seem to be important in the early stages of infection by the mycorrhizal fungi.

From the present results it is not possible to conclude how much of these volatile organic metabolites of the host and the fungal symbiont is actually available outside the ectomycorrhizal root systems to act upon the root pathogens. However, this investigation indicates what potentialities to resist pathogens Scots pine and *Boletus variegatus* might have during their association.

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